(54) Title: AN ASSAY FOR DETECTING INHIBITORS OF THE ENZYME MUELOPEROKIDASE

(57) Abstract: An assay for detecting inhibitors of the enzyme myeloperoxidase said method comprising: reacting myeloperoxidase with hydrogen peroxide and a chloride source to generate hypochlorus acid in the presence of a potential inhibitor of said myeloperoxidase; reacting any formed hypochlorous acid with a suitable amine to form the corresponding chloramine; Optionally removing any unreacted hydrogen peroxide; reacting any formed chloramine with a suitable detector molecule in the presence of iodide to form oxidised detector molecule; determining the amount of formed oxidised detector molecule by measuring the absorbance or fluorescence at a suitable wavelength; Identifying as inhibitors of myeloperoxidase those compounds which cause the amount of formed oxidised detector molecule to be decreased. And for use in diagnostic tests for myeloperoxidase activity is disclosed.

An assay for detecting inhibitors of the enzyme mueloperokidase

Background of the invention

5   Resistance to infection by microorganisms involves both non-specific mechanisms and
     adaptive immune responses mediated by B and T lymphocytes. Non-specific mechanisms
     such as enzyme action, pH and epithelial tissue secretions prevent invasion by many
     organisms. However, when the first line of defence is breached, phagocytes engulf
     infectious agents and process them leading to the stimulation of B and T cells.

10   Deficiencies in the phagocytic system, whether hereditary or acquired, have serious
     consequences, since microorganisms with low pathogenicity for normal individuals can
     cause recurrent infections in individuals with ineffective phagocytic systems.

     Polymorphonuclear leukocytes (PMNs) are of particular importance for combating

15   infections. These cells contain an enzyme, myeloperoxidase, with well documented
     microbicidal action. PMNs act non-specifically by phagocytosis to engulf
     microorganisms, incorporate them into vacuoles, termed phagosomes, which fuse with
     granules containing myeloperoxidase to form phagolysosomes. In phagolysosomes the
     enzymatic activity of the myeloperoxidase leads to the formation (from hydrogen peroxide

20   and chloride) of hypochlorous acid, a potent bactericidal compound. Hypochlorous acid is
     oxidizing in itself, and reacts most avidly with thiols and thioethers but also converts
     amines into chloramines and chlorinates aromatic amino acids. Macrophages are large
     phagocytic cells which, like PMNs are capable of phagocytosing microorganisms.
     Macrophages can generate hydrogen peroxide and upon activation also produce

25   myeloperoxidase. In addition, myeloperoxidase in the plasma is taken up by macrophages.

     Linkage of myeloperoxidase activity to disease has been implicated in numerous
     inflammatory diseases including Alzheimer's disease, multiple sclerosis, asthma,
     atherosclerosis, cancer, cystic fibrosis, chronic obstructive pulmonary disease,

30   inflammatory bowel disease, and rheumatoid arthritis. Assays for identification of

inhibitors of myeloperoxidase will be invaluable in demonstrating the contribution of hypochlorous acid to the pathogenesis of such diseases, as well as for diagnostic tools. They will also provide the platform for the development of effective drugs against myeloperoxidase-dependent tissue damage. A suitable screening assay for MPO inhibitors should measure the chlorination activity of myeloperoxidase under realistic physiological conditions. It must be simple, sensitive, and produce a coloured or fluorescent product, and it must not perturb the activity of MPO. The detector will need to be present at high enough concentrations to scavenge all the hypochlorous acid produced but not so as to react directly with the enzyme. Currently, the most commonly used assay is based on the primary formation of taurine chloramine from hypochlorous acid and taurine. Previous attempts to use modified forms of this taurine chloramine assay for measuring the activity of myeloperoxidase have revealed specific problems that negate its use for screening potential inhibitors of the enzyme. Major problems are that dimethylsulphoxide (DMSO), the solvent in which test compounds are most conveniently and usually dissolved, interferes with the assay, and many of the test compounds absorb in the same region as the commonly used chromophore.

Physiologically, PMN-derived myeloperoxidase primarily catalyzes the hydrogen peroxide-dependent oxidation of halides such as chloride ions, but its ability to use electron donors such as O-dianisidine as a substrate forms the basis of an assay to quantify PMNs in inflamed tissue. Adaptations of the method to avoid artefacts caused by tissue/blood constituents, such as the cytosolic enzymes, catalase and glutathione peroxidase, reducing substrates such as glutathione and ascorbic acid, and heme proteins have been described (Grisham et al., Methods in Enzymology, Vol. 186, Ed. Packer et al., San Diego: Academic Press, pp. 729-742 (1990)). However, there is a need for a more reliable analytical method of quantifying the PMN content of inflamed tissues that is not affected by other endogenous tissue constituents.

The present invention describes a simple, sensitive and robust assay that avoids the major problems associated with currently known assays and that is well suited to the

identification of inhibitors of myeloperoxidase. In another aspect, the present invention is useful for certain diagnostic tests associated with myeloperoxidase.

Description of the Invention

In a first aspect, the present invention provides a method for identifying inhibitors of myeloperoxidase (MPO), the method comprising:

reacting myeloperoxidase with hydrogen peroxide and a chloride source to generate hypochlorous acid in the presence of a potential inhibitor of said myeloperoxidase;

reacting any formed hypochlorous acid with a suitable amine to form the corresponding chloramine;

optionally removing any unreacted hydrogen peroxide;

reacting any formed chloramine with a suitable detector molecule in the presence of iodide to form oxidised detector molecule;

determining the amount of formed oxidised detector molecule by measuring the absorbance or fluorescence at a suitable wavelength;

identifying as inhibitors of myeloperoxidase those compounds which cause the amount of formed oxidised detector molecule to be decreased.

In one embodiment of the above invention, the amine used is taurine which is converted by hypochlorous acid into taurine chloramine. A suitable concentration of taurine is about 5 to 10 mM.

In another embodiment, any unreacted hydrogen peroxide is removed using catalase. A suitable concentration of catalase is about 10 to 20 µM.

Sodium chloride may be used as a suitable source of chloride, for example, in an amount of about 50 to 150 mM.

Hydrogen peroxide may be added as such, or may be generated *in situ* using, for example, enzymatic generation of hydrogen peroxide by an oxidase such as glucose oxidase or xanthine oxidase. A suitable concentration of hydrogen peroxide is, for example, 10 to 100 μM.

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In another embodiment, the detector molecule used is a benzidine derivative. A preferred benzidine derivative is 3,3',5,5'-tetramethylbenzidine (TMB), and the formed oxidised 3,3',5,5'-tetramethylbenzidine is assayed by measuring the absorbance at 645 to 650 nM.

10   In alternative embodiments, the detector molecule is a fluorescent probe such as one of the molecules shown in Figure 1.

Figure 1

15
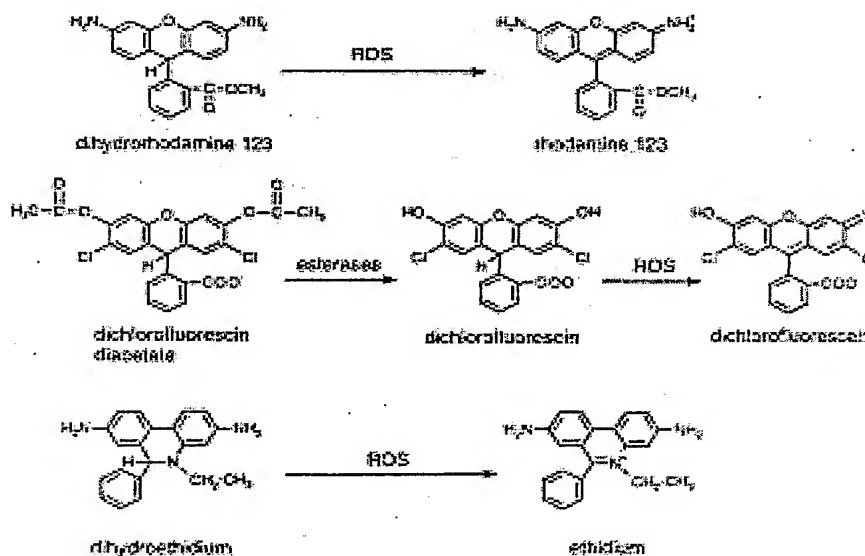
For example, dihydrorhodamine may be used as the detector molecule. This non-fluorescent compound is oxidized to rhodamine, which emits fluorescence at 536 nm when exited at 500 nm.

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More particularly, the method of the present invention comprises:

reacting myeloperoxidase with hydrogen peroxide to generate hypochlorous acid in the presence of a potential myeloperoxidase inhibitor and reacting any formed hypochlorous acid with taurine to form taurine chloramine in a suitable buffer containing sodium chloride and 10 mM taurine; ·

5   optionally adding catalase to a concentration of up to about 20 μg/mL to destroy any residual hydrogen peroxide;

reacting any formed taurine chloramine with 3,3',5,5'-tetramethylbenzidine in the presence of potassium iodide to form oxidised 3,3',5,5'-tetramethylbenzidine in a suitable buffer containing 3,3',5,5'-tetramethylbenzidine and 10 to 20% dimethylformamide; and

10   measuring the amount of formed oxidised 3,3',5,5'-tetramethylbenzidine.

Buffers that may be used include suitable acetate, citrate or phosphate buffers of pH 5 to pH 8. The buffer should be present at a concentration of at least 10 mM, preferably at least 20 mM. Preferably the reaction with potassium iodide is performed at about pH 5.4

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Thus, in one particular embodiment, the method of the present invention comprises:

reacting myeloperoxidase (2.5 nM) with hydrogen peroxide (100 μM) and sodium chloride (140 mM) in 20 mM phosphate buffer pH 6.5 to generate hypochlorous acid in the presence of a potential myeloperoxidase inhibitor (in compound solvent, DMSO, at 1%)

20   and reacting any formed hypochlorous acid with 10 mM taurine to form taurine chloramine;

stopping and developing the assay by adding, as final concentrations, glacial acetic acid (400 mM), potassium iodide (100 μM) and 3,3',5,5'-tetramethylbenzidine in dimethylformamide (10 mM); and

25   measuring the amount of formed oxidised 3,3',5,5'-tetramethylbenzidine.

In another particular embodiment, the method of the present invention comprises:

· reacting myeloperoxidase (5 to 20 nM) with hydrogen peroxide (100 μM) to generate hypochlorous acid in the presence of a potential myeloperoxidase inhibitor and reacting

any formed hypochlorous acid with taurine to form taurine chloramine in 20 mM pH 6.5

phosphate buffer containing 100 mM sodium chloride and 10 mM taurine;

adding catalase to a concentration of 20 µg/mL to destroy any residual hydrogen

peroxide;

5   reacting any formed taurine chloramine with 3,3',5,5'-tetramethylbenzidine in the

presence of 200 µM potassium iodide to form oxidised 3,3',5,5'-tetramethylbenzidine in

200 mM sodium acetate buffer pH 5.4 containing 2 mM 3,3',5,5'-tetramethylbenzidine, and

10 to 20% dimethylformamide; and

measuring the amount of formed oxidised 3,3',5,5'-tetramethylbenzidine.

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In another particular embodiment, the method of the present invention comprises:

reacting myeloperoxidase (2.7 nM) with hydrogen peroxide (50 µM) in 20 mM

phosphate buffer pH 6.5 containing 140 mM sodium chloride and 10 mM taurine in the

presence of a potential myeloperoxidase inhibitor; this assay solution (150 µL) was then

15   added to each well of a 96 well plate;

stopping and developing the reaction by adding to each well 50 µL of a reagent

comprising sodium iodide (100 µM), acetic acid (400 mM) and 10 mM TMB in 50%

dimethylformamide; and

measuring the amount of formed oxidised 3,3',5,5'-tetramethylbenzidine.

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In another particular embodiment, the method of the present invention uses an assay buffer

comprising 20 mM sodium/potassium phosphate buffer pH 6.5 containing 10 mM taurine

and 100 mM NaCl, and a developing reagent comprising 2 mM TMB, 200 µM KI, 200

mM acetate buffer pH 5.4 with 20 % DMF.

25

Further particular embodiments are described in the Examples section below.

The present invention overcomes two specific problems that precluded use of known

taurine chloramine assays for measuring the activity of myeloperoxidase as a way of

30   screening for potential enzyme inhibitors. Firstly, a commonly used solvent,

dimethylsulphoxide, interfered with these assays, and, secondly, many test compounds absorbed optically in the same region as the chromophore.

The present invention is a modified taurine chloramine assay in which the interference by
5   dimethylsulphoxide is minimized and the detection process is free of optical interference by test compounds.

Inhibition of purified myeloperoxidase by test compounds has been measured using the modified assay establishing that the modified assay is useful for assessing the ability of
10   compounds to inhibit myeloperoxidase. Further, the present invention meets the requirements necessary for high-throughput screening (HTS). Still further, the invention provides a method for detecting inhibitors of myeloperoxidase useful for identifying compounds with potential for treating subjects suffering from diseases in which MPO activity is implicated.

15

It has been found that oxidation of a suitable detector molecule such as 3,3',5,5'-tetramethylbenzidine by taurine chloramine catalysed by iodide at a suitable pH provides an assay which suffers from minimal interference by dimethylsulphoxide (DMSO). When the detector molecule is 3,3',5,5'-tetramethylbenzidine, a blue product is formed having an
20   absorbance maximum at about 645 nm.

The assay of the present invention is a sensitive and specific method for detecting hypochlorous acid, HOCl. It is also useful for determining the ability of test compounds to inhibit myeloperoxidase. It is particularly useful for HTS to identify inhibitors of
25   myeloperoxidase.

For the particular embodiment disclosed in Example 3 below, it has been found that 20 mM pH 6.5 phosphate buffer containing 100 mM sodium chloride and 10 mM taurine (RNH_2) (hereinafter PBST) minimizes the inhibition of myeloperoxidase by hydrogen
30   peroxide, and a concentration of taurine of 10mM has been found to be sufficient to

ensure that all hypochlorous acid (HOCl) generated (see Reaction 1, below) is trapped as taurine chloramine (RNHCl) (see Reaction 2, below). Taurine chloramine has been found to be stable at room temperature for at least an hour. Therefore, to minimize potential consumption of taurine chloramine by reaction with test compounds, the assay has been

5 designed so that the amount generated is about 5-10 times greater than the concentration of the test compound.

Reaction 1: H_2O_2 + Cl^- in the presence of MPO $\rightarrow$ HOCl + H_2O

10 Reaction 2: HOCl + RNH_2 $\rightarrow$ RNHCl + H_2O

TMB may be used as the detector molecule. It is used at a final concentration of about 1 mM so that it is present at a vast excess over oxidant, which ensures a 1:1 stoichiometry for its reaction with taurine chloramine. The addition of potassium iodide is essential

15 because it catalyses the reaction between taurine chloramine and TMB, and no reaction occurs in the absence of iodide (see Reactions 3 and 4, below). A pH of about 5.4 has been found to be optimal to maintain the stability of oxidized TMB and allow taurine chloramine to oxidize iodide. At a lower pH, non-specific oxidation of iodide can occur and at a higher pH, oxidation of iodide is slow and oxidized TMB is less stable.

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Reaction 3: RNHCl + I^- + H_2O + H^+ $\rightarrow$ RNH_2 + HOI

Reaction 4: HOI + TMB $\rightarrow$ TMB_{ox} + I^- + H_2O

25 The taurine chloramine assay of the present invention comprises two parts, one, a process to generate taurine chloramine and, two, a process to oxidize a detector molecule such as TMB. In the first reaction, myeloperoxidase forms hypochlorous acid which is reacted with taurine to become trapped as taurine chloramine. In the second reaction, the formed taurine chloramine is used to quantitatively oxidise, for example, TMB to form oxidized

30 TMB.

As a way of validating the above assay, taurine chloramine was generated by adding reagent HOCl to 5.5 mM taurine in 10 mM phosphate buffer pH 7.4 containing 140 mM sodium chloride. When an equal volume of developing reagent was added to the taurine

5 chloramine the solution turned blue. The absorption spectrum of product has a broad peak between 500 and 700 nm with the maximum at 645 nm. The extinction coefficient was determined to be $14,520 \pm 330$ M^{-1} cm^{-1}. The absorption at 645 nm was found to be directly proportional to the concentration of HOCl added to the PBST.

10 The optimal concentration of iodide was determined by employing a range of concentrations of iodide from 0 to 1 mM. Maximum formation of oxidized TMB was found to occur at iodide concentrations of greater than 10 μM.

It is known that DMSO interferes with the taurine chloramine assay when the oxidation of

15 TMB is promoted by lowering the pH to 1. Therefore, inhibition of the oxidation of TMB by DMSO in the assay of the present invention was measured. To measure inhibition, a range of concentrations of DMSO was added to PBST (pH 7.4) before adding 56 μM HOCl. The reaction was permitted to proceed for 10 minutes before adding an equal volume of developing reagent. It was found that DMSO had only a minor effect on the

20 detection of taurine chloramine provided that the concentration of DMSO was below about 1% in the PBST.

The taurine chloramine assay of the present invention was used to detect HOCl generated by purified human myeloperoxidase. Hydrogen peroxide (100 μM) was added to 20 mM

25 phosphate buffer pH 6.5 containing 20 nM myeloperoxidase, 100 mM NaCl and 10 mM taurine. The mixture was incubated for 15 minutes at room temperature and the reaction stopped by adding catalase (10 μg/ml). Five minutes later developing reagent was added to detect the generated taurine chloramine. The amount of taurine chloramine produced was compared to that formed by adding 100 μM HOCl to the PBST. By comparison with

30 the HOCl standard, it was shown that 100 μM hydrogen peroxide was converted into 113

μM HOCl. Therefore, within experimental error, all the hydrogen peroxide had been converted into HOCl and then trapped as taurine chloramine. The concentration of stock hydrogen peroxide was determined using E_{240} 43.6 M^{-1} cm^{-1} and that of stock hypochlorite using E_{292} 350 M^{-1} cm^{-1}.

Studies were performed to determine whether any oxidation of TMB occurs in the absence of chloride. In other words, to show that oxidation of TMB is limited to the original formation of HOCl, and it does not occur through direct oxidation by hydrogen peroxide and myeloperoxidase. Oxidation of TMB was not observed in the absence of chloride and further studies with catalase support this observation.

The ability of various compounds to affect the chlorination activity of myeloperoxidase confirmed that the assay of the present invention was measuring HOCl production, and that the assay is suitable for revealing potential inhibitors of the enzyme.

Hypochlorous acid was generated by myeloperoxidase in the absence or presence of 10 μM test compounds under the conditions described above for the time course experiment. Reactions were initiated by adding hydrogen peroxide and stopped five minutes later by adding catalase. Addition of catalase at 20 μg/ml achieved a 90% inhibition of the formation of HOCl.

All of the above assays may also optionally be run in the presence of tyrosine. A suitable concentration of tyrosine is about 5 to 20 μM.

A time course experiment showed that tyrosine enhanced production of HOCl because hydrogen peroxide was inhibiting myeloperoxidase. This experiment also showed that the chlorination activity of myeloperoxidase is being measured because tyrosine does not affect the peroxidation activity. This conclusion is supported by the effect of dapsone which is known to inhibit the chlorination activity but not the peroxidation activity of myeloperoxidase. Further experiments showed that dapsone, ABAH, p-bromoaniline, and

dimethoxybenzene inhibited the production of HOCl to a similar extent as has been shown
in other assays for the chlorination activity of myeloperoxidase. Thus, the assay of the
present invention is suitable for revealing inhibitors of myeloperoxidase. Experiments also
showed that DMSO did not affect the detection of HOCl. The results are presented in
Table 1.

Table 1. Inhibitors of HOCl production by purified human myeloperoxidase.

Inhibitor	[HOCl] (μM)	% Inhibition
None	56.2 ± 2.1	0
Dapsone	1.8 ± 0.1	97
ABAH	5.5 ± 1.1	90
Tyrosine	107.0 ± 0.5	-90
p-Bromoaniline	2.5 ± 0.1	95.6
1,4-Dimethoxybenzene	15.2 ± 1.0	73
Catalase	8.8 ± 1.3	84
DMSO (0.1%)	52.8 ± 1.2	6

In another aspect, the invention relates to the use of an assay method essentially as
described above to measure MPO activity levels, particularly in biological fluids.
Myeloperoxidase activity has been implicated in numerous inflammatory diseases
including Alzheimer's disease, multiple sclerosis, asthma, atherosclerosis, cancer, cystic
fibrosis, chronic obstructive pulmonary disease, inflammatory bowel disease, and
rheumatoid arthritis. The assay of the present invention may thus be used to look for MPO
as a risk factor in a wide range of inflammatory diseases, including neuroinflammatory
diseases.

Neutrophils and other phagocytes manufacture O_2^- (superoxide) by the one-electron reduction of oxygen at the expense of NADPH. Most of the O_2^- reacts with itself to form H_2O_2 (hydrogen peroxide). From these agents a large number of highly reactive microbicidal oxidants are formed, including HOCl (hypochlorous acid), which is produced by the myeloperoxidase-catalyzed oxidation of Cl^- by H_2O_2; OH (hydroxyl radical), produced by the reduction of H_2O_2 by Fe^{++} or Cu^+; $ONOO^-$ (peroxynitrite), formed by the reaction between O_2^- and NO ; and many others. These reactive oxidants are manufactured for the purpose of killing invading microorganisms, but they also inflict damage on nearby tissues, and are thought to be of pathogenic significance in a large number of diseases (B. M. Babior, Am. J. Med., 2000, **109**, 33-44). Included among these are Alzheimer's disease, multiple sclerosis, asthma, atherosclerosis, cancer, cystic fibrosis, chronic obstructive pulmonary disease, inflammatory bowel disease, rheumatoid arthritis, emphysema, acute respiratory distress syndrome, reperfusion injury and malignancy.

MPO is also implicated in coronary artery disease (Hazen et al, JAMA, 2001, **286**, 2136-2142). Thus, the assay of the present invention may be used to look for MPO in peripheral blood neutrophils as a risk factor for atherosclerosis.

Assays for the identification of inhibitors of myeloperoxidase will be invaluable in demonstrating the contribution of hypochlorous acid to the pathogenesis of such diseases, as well as for diagnostic tools. They will also provide a platform for the development of effective drugs against myeloperoxidase-dependent tissue damage.

The invention is now illustrated by means of the following non-limiting examples.

Example 1

Here we describe an *in vitro* MPO assay that was developed to assess inhibition of enzyme activity. Essentially the MPO assay was designed to measure the production of

hypochlorous acid (HOCl), which is the key physiological product generated by the enzyme *in vivo*. An outline of the assay reactions is given:

                     MPO
5   1. $H_2O_2 + Cl^- $ -------------> $HOCl + H_2O$

    2. $HOCl + RNH_2$ (taurine) -------------> RNHCl (taurine chloramine) + H_2O

    3. $RNHCl + I^- + H_2O$ --------------------> $RNH_2 + HOI$ or ICl
10   H+
    4. HOI (or ICl) + TMB ----------------------------> TMB (oxidised) + $I^- + H_2O$
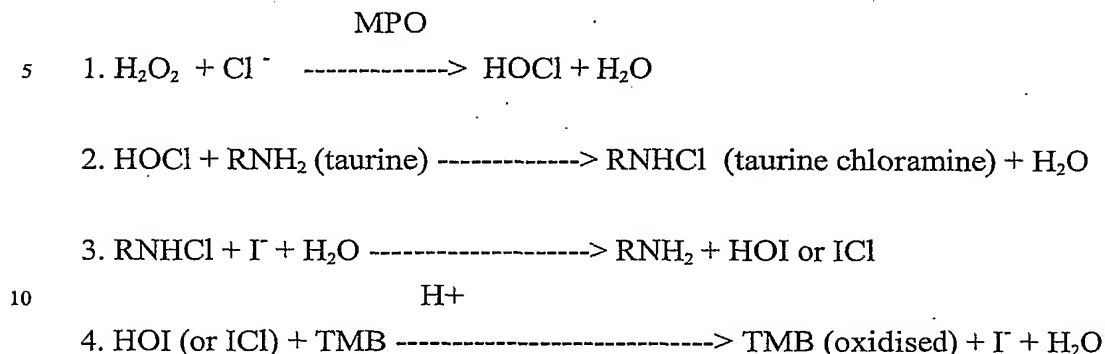
The reaction mixtures in 20mM phosphate buffer pH 6.5 contained 2.5nM MPO (purified human enzyme from Planta), 100µM H_2O_2, 140 mM NaCl, 10 mM taurine, 20µM tyrosine

15 and compound solvent, DMSO, at 1%. Compounds were preincubated with the MPO enzyme in buffer for about 15 minutes prior to start of the reaction with H_2O_2. The whole reaction was carried out at room temperature for 10 minutes in a 96-well plate. The reaction was terminated by a stop/developing reagent, which consisted, in their final concentrations, of glacial acetic acid (400 mM), KI (100 µM) and TMB in

20 dimethylformamide (10 mM). All test concentrations were done in duplicate with at least two separate determinations (n=2, unless otherwise stated). The inhibitory concentration for a compound is presented as pIC_{50}, which is $-\log IC_{50}$.

Various compounds have been tested against the human MPO. It can be seen that dapsone

25 is the most potent inhibitor of the sulfones/sulfonamides tested. Indoles and other compounds are also effective in blocking the production of HOCl by human MPO. All data obtained for the sulfones/sulfonamides, indoles and miscellaneous compounds are presented in Tables 2, 3 and 4, respectively.

30

Table 2: Inhibition of human MPO-HOCl production by sulfones/sulfonamides

Compound	pIC$_{50}$
Dapsone	6.2
N-1(2-thiazolyl)-sulfanilamide	6.0
Sulfanilamide	6.0
Sulfapyridine	5.7
Sulfaguanidine	5.5
Sulfisoxazole	5.2
Sulfadiazine	5.2
Sulfanitran	5.1

Table 3: Inhibition of human MPO-HOCl production by indoles

Compound	pIC$_{50}$
5-Methoxytryptophol	6.3
5-Methoxytryptamine	6.2
Melatonin	6.1
3-Methylindole	5.9
6-Methoxyindole	5.8
Indole	5.7
5-Methoxyindole	5.6

Table 4: Inhibition of human MPO-HOCl production by miscellaneous compounds

Compound	pIC$_{50}$
Ethyl aminobenzoate	6.2
3-Aminobenzoic acid ethyl ester	6.2
p-Aminobenzamidine	5.6
Piroxicam	5.6 (n=1)
Diclofenac	5.4
Vanillin	5.1

Example 2

Here we describe the use of a functional human neutrophil assay to determine the effects of

MPO inhibitors on the production of HOCl. This assay detects the production of HOCl

5   from stimulated (e.g. PMA, LPS, fMLP, zymozan) human neutrophils. Human neutrophils

were purified from fresh heparinised blood by density centrifugation on Polymorphprep

(Nycomed). These neutrophils were used immediately after purification. A standard

reaction mixture contained the following: 2 x 106 neutrophils, 140 mM NaCl, 5 mM

taurine, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mg/ml glucose. Test compounds were made up

10   in DMSO and added to cells, with a final DMSO concentration of 0.5%. Test compounds

were given 15 minutes preincubation at 37° C with neutrophils prior to the addition of the

PMA stimulant (1 μg/ml). The assay was then allowed to progress for another 30 minutes

at 37° C. At the end of the incubation, supernatants were collected by centrifugation and

assayed for HOCl by using the stop/development reagent as above. All compounds were

15   tested in duplicate with at least two separate determinations, n=2 from two different

donors.

The data for some of these inhibitors are shown in Table 5.

20   Table 5: Inhibition of HOCl production by stimulated human neutrophils

HOCl production by neutrophils	pIC_{50}
Primaquine	4.9
Sufanilamide	4.8
Dapsone	4.7
Sulfapyridine	4.5

We have also shown that under the assay conditions and concentrations of inhibitors used,

human neutrophils were not affected by cytotoxicity, as assessed by the release of lactate

25   dehydrogenase from damaged neutrophils. Lactate dehydrogenase activity was measured

as described by Boehringer Mannheim GmbH, Sandhofer Strabe 116, D-68305 Mannheim, Germany (Cytotoxicity Detection Kit-LDH- Cat No: 1 644 793).

Example 3

In this example, the assay is performed by reacting myeloperoxidase (5 to 20 nM) with hydrogen peroxide (100 μM) to generate hypochlorous acid in the presence of a potential myeloperoxidase inhibitor and reacting any formed hypochlorous acid with taurine to form taurine chloramine in 20 mM pH 6.5 phosphate buffer containing 100 mM sodium chloride and 10 mM taurine. Catalase is then added to a concentration of 20 μg/mL to destroy any residual hydrogen peroxide. An approximately equal volume of a developing reagent of 200 nM sodium acetate buffer pH 5.4 containing 2 mM TMB, 200 μM potassium iodide and 10 to20% dimethylformamide is then added. The amount of oxidised 3,3',5,5'-tetramethylbenzidine formed is then measured after about 5 minutes using absorbance spectroscopy at about 645 nM.

Example 4

An assay was run essentially as described in Example 3 but:

        reacting myeloperoxidase (2.7 nM) with hydrogen peroxide (50 μM) in 20 mM phosphate buffer pH 6.5 containing 140 mM sodium chloride and 10 mM taurine in the presence of a potential myeloperoxidase inhibitor; this assay solution (150 μL) was then added to each well of a 96 well plate;

        stopping and developing the reaction by adding to each well 50 μL of a reagent comprising sodium iodide (100 μM), acetic acid (400 mM) and 10 mM TMB in 50% dimethylformamide; and

        measuring the amount of formed oxidised 3,3',5,5'-tetramethylbenzidine.

The sensitivity and stability of the assay as described in Example 4 was established as follows. Varying concentrations of HOCl were added to 10 mM phosphate buffer

containing 10 mM taurine to generate taurine chloramine. 150μl of the solutions were
added to 96 well plates followed by 50 μl of developing reagent. Plates were read (A_{650})
immediately after mixing and again at 1 hour to determine sensitivity and product stability.
The results are shown in Figure 2.
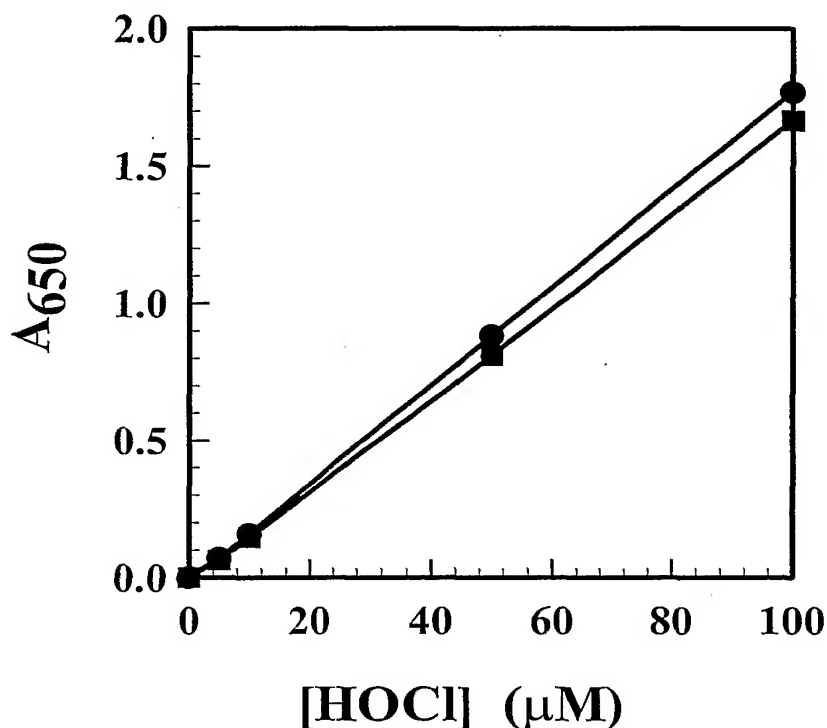
Figure 2. Standard curve for detection of HOCl using the Iodide Catalysed TMB assay.
A_{650} was read immediately after mixing (●) and one hour later (■).

From Figure 2 it is can be seen that the TMB assay can easily detect 100 μM HOCl when
performed in a 96 well plate. The product is very stable with less than 10% decaying at all
concentrations after an hour at room temperature.

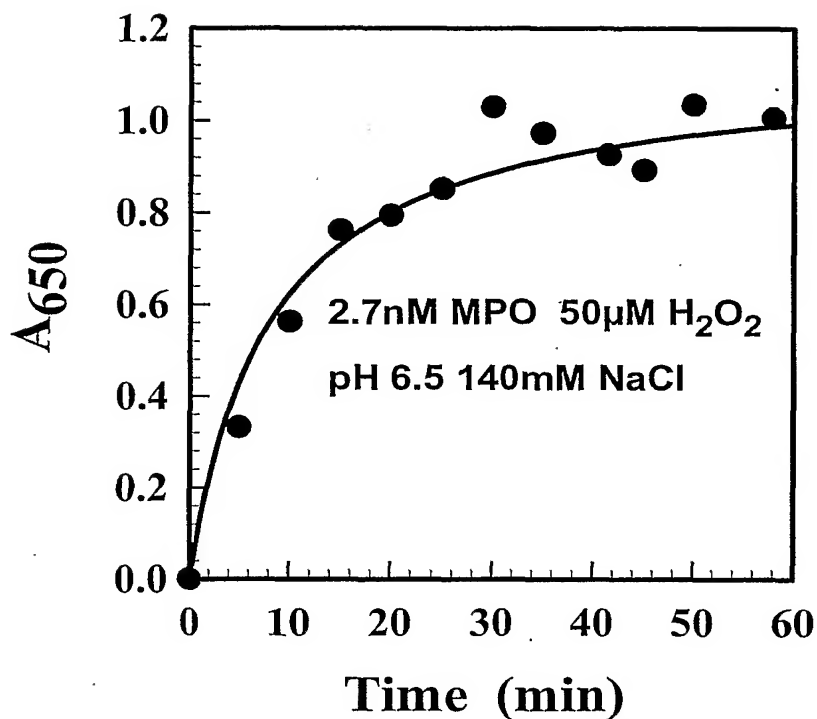The time course for the production of HOCl by MPO is shown in Figure 3:

Figure 3. Time course for production of HOCl by MPO

From Figure 3 it is apparent that conversion of H_2O_2 to HOCl took about 30 minutes under
the reaction conditions described above. The shape of the progress curve indicates that
there was little inhibition by H_2O_2. By comparing Figs 2 & 3 it is evident that MPO
converted all the H_2O_2 into HOCl. There was thus no need to add any catalase to remove
excess H_2O_2.

The colour yield for the reaction at 5 minutes increased by only 6% over the following
hour. This indicates that addition of developing reagent prevents MPO from producing
more HOCl and from directly oxidizing TMB.

Example 5

Assay buffer: 20 mM sodium/potassium phosphate buffer pH 6.5 containing 10 mM
taurine and 100 mM NaCl.

Developing reagent: 2 mM TMB, 200 μM KI, 200 mM acetate buffer pH 5.4 with 20 %
DMF.

To 10 μl of diluted compounds in assay buffer, 40 μl of MPO (final concentration 2.5 nM)
was added for 10 minutes at room temperature. Then 50 μl of H_2O_2 (final concentration
100 μM), or assay buffer alone as a control, were added for 10 minutes at room
temperature. The reaction was stopped by adding 10 μl 0.2 mg/ml of catalase (final
concentration 18 μg/ml) for 5 minutes before 100 μl of TMB developing reagent was
added (2 mM TMB in 200 mM acetate buffer pH 5.4 containing 20% dimethylformamide
(DMF) and 200 μM KI). Plates were mixed and subsequently read at 650 nm.

Using the assay as described in Example 5, the reference compound dapsone showed an
IC_{50} value of 257 ± 73 nM in the absence of tyrosine and 7120 ± 610 nM in the presence of
8 μM tyrosine. A further reference compound, 4-aminobenzoic acid hydrazide (ABAH),
showed an IC_{50} value of 86 ± 6 nM in the absence of tyrosine and 118 ± 8 nM in the
presence of 8 μM tyrosine.

Example 6

This example describes an assay using dihydrorhodamine as the detector molecule that is
suitable for high throughput screening (HTS) using 384 well plates.

Assay construction –

Enzyme solution	110ml of 200mM NaH_2PO_4 pH 6.5	(20mM final)
	110ml of 1.4M NaCl	(140mM final)
	110ml of 100mM Taurine	(10mM final)
	2.75ml of 10mM Tyrosine	(25μM final)
	275μl of 10μM MPO	(25nM final)

made up to 1100 ml distilled water

Substrate solution   110ml of 200mM NaH_2PO_4 pH 6.5   (20mM final)

110ml of 1.4M NaCl   (140mM final)

110ml of 100mM Taurine   (10mM final)

20µl of 8.8M H_2O_2 (160 µM final)

made up to 1100 ml distilled water

Detection solution   110ml of 200mM NaH_2PO_4 pH 6.5   (20mM final)

110ml of 1.4M NaCl   (140mM final)

110ml of 100mM Taurine   (10mM final)

220ml DMSO   (20% final)

16.5ml of 20mM KI   (300µM final)

8ml of 72.5mM Dihydrorhodamine   (527.3 µM final)

made up to 1100 ml distilled water

Negative control   20ml of 10mM Dapsone   (1mM final)

made up to 200 ml distilled water

Compounds dilutions are prepared using 5% $DMSO/H_2O$, to provide a compound concentration of 110µM in 5µl.

Positive and negative controls contain 0.4µl DMSO vehicle and are diluted as with compound. Upon completion of the diluted plate, 5µl of 1 mM of the reference compound dapsone was added to negative control wells.

Procedure

25 µl of the enzyme solution was added to all wells per plate;

Plates are then pre-incubated for 45 minutes;

25 µl of the substrate solution was added to all wells per plate;

Plates are then incubated for 15 minutes;

25 µl of the detection solution was added to all wells per plate;

The plates were counted by reading fluorescent intensity (excitation 485 nm, emission 530 nm, dichroic 505 nm).

Example 7

Assay for diagnostic use

Assays of the MPO activity may also be performed on MPO containing tissue like blood, cellular fractions of blood, sputum or isolated tissue with an acute inflammation. For this type of assay it is preferred to use glucose oxidase to generate the hydrogen peroxide at about 10 µM/min and then stop the reaction after 5 to 10 minutes with the developing reagent. In this way the assay provides a simple diagnostic test and the information thus obtained can be widely used to assess MPO as a risk factor in numerous inflammatory diseases.

Claims

1. A method for identifying inhibitors of myeloperoxidase (MPO), said method comprising:

reacting myeloperoxidase with hydrogen peroxide and a chloride source to generate hypochlorous acid in the presence of a potential inhibitor of said myeloperoxidase;

reacting any formed hypochlorous acid with a suitable amine to form the corresponding chloramine;

optionally removing any unreacted hydrogen peroxide;

reacting any formed chloramine with a suitable detector molecule in the presence of iodide to form oxidised detector molecule;

determining the amount of formed oxidised detector molecule by measuring the absorbance or fluorescence at a suitable wavelength;

identifying as inhibitors of myeloperoxidase those compounds which cause the amount of formed oxidised detector molecule to be decreased.

2. A method according to Claim 1 wherein the detector molecule is 3,3',5,5'-tetramethylbenzidine (TMB).

3. A method according to Claim 1 or Claim 2 wherein the amine is taurine.

4. A method according to Claim 3 wherein the reaction of 3,3',5,5'-tetramethylbenzidine (TMB) with the taurine chloramine in the presence of iodide is conducted at about pH 5.4.

5. A method according to any one of the Claims 1 to 4 wherein any unreacted hydrogen peroxide is removed using catalase.

6. A method according to any one of Claims 1 to 5 that is performed as a high throughput screen.

7.   Use of a method according to any one of Claims 1 to 6 for use in a diagnostic test for myeloperoxidase activity.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 02/00877

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/28 // C12N 9/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q, G01N, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI-DATA, EPO-INTERNAL, BIOSIS, MEDLINE, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biochemical Pharmacology, Volume 44, no. 3, 1992, Paula M. Bozeman et al: "Inhibition of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase by dapsone", page 553 - page 563	1-7
A	Biophys, Volume 296, 1992, Anthony J. Kettle et al: "Assays for the Chlorination Activity of Myeloperoxidase", page 502 - page 513	1-7
A	Am. J. Physiol., Volume 251, 1986, M. B. Grisham et al: "Assessment of Leukocyte Involvement during Ischemia and Reperfusion of Intestine", page 729 - page 743	1-7

X	Further documents are listed in the continuation of Box C.		See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Analytical Biochemistry, volume 132, 1983, Kazuo Suzuki et al: "Assay Method for Myeloperoxidase in Human Polymorphonuclear Leukocytes", page 345 - page 352	1-7